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<p>(54) Title: CLONING AND CHARACTERIZATION OF NAPSIN, AN ASPARTIC PROTEASE</p> <p>(57) Abstract</p> <p>A previously unknown aspartic protease capable of cleavage of proteins by hydrolysis, referred to herein as "napsin", has been cloned from a human liver library. Two cDNA clones have been cloned, sequenced and expressed. These encode isozymes of the protease, referred to as "napsin A" and "napsin B". The gene has also be obtained and partially sequenced. A process for rapid purification of the enzyme using immobilized pepstatin has also been developed, and enzyme isolated from human kidney tissue. Polyclonal antibodies to the enzymes have been made which are also useful for isolation and detection of the enzyme. Similarities to other aspartic proteases, especially cathepsin D, establish the usefulness of the enzyme in diagnostic assays as well as a protease. Either or both the amount or type of napsin expressed in a particular tissue can be determined using labelled antibodies or nucleotide probes to the napsin.</p>		

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## CLONING AND CHARACTERIZATION OF NAPSIN, AN ASPARTIC PROTEASE

## Background of the Invention

The present invention relates to a previously unknown aspartic protease present in human liver, isolated by cloning of a gene from a human liver cDNA library.

Members of the aspartic protease family are characterized by the presence of catalytic aspartic acid residues in their active center. There are five aspartic proteases known to be present in human body. Pepsin and gastricsin are secreted into the stomach for food digestion. Gastricsin is also present in the seminal plasma. Cathepsin D and cathepsin E are present intracellularly to carry out protein catabolism. Renin, which is present in the plasma, is the key enzyme regulating the angiotensin system and ultimately the blood pressure.

Eukaryotic, including human, aspartic proteases are homologous in protein and gene sequences, but have different amino acid and nucleotide sequences. The cDNA and genes of all five human aspartic proteases have been cloned and sequenced. They are synthesized as a single chain zymogen of about 380 residues, which are either secreted or directed to intracellular vacuoles. Upon activation by a self-catalyzed process (except prorenin), an N-terminal *pro* segment of about 45-residues is cleaved off to produce mature enzymes (Tang and Wong, J. Cell. Biochem. 33, 53-63 (1987)). In some cases, for example, with cathepsin D and renin, mature proteases are further cut into two chains. The three-dimensional structures of the aspartic proteases are very similar. Each enzyme contains two internally homologous lobes (Tang *et al.*, Nature 271, 618-621 (1978)). The active-site cleft, which can accommodate eight substrate residues, and two catalytic aspartic acids, are located between the lobes.

These proteases have distinct and important physiological roles. In addition to their importance in physiological functions, these enzymes are also associated with pathological states. For example, human pepsin and

gastricsin are diagnostic indicators for stomach ulcer and cancer (Samloff, Gastroenterology 96, 586-595 (1989); Miki *et al.*, Jpn. J. Cancer Res. 84, 1086-1090 (1993)). Cathepsin D is located in the lysosome. Its main function is the catabolism of tissue proteins. Recent evidence from mice  
5 without a functional cathepsin D gene, however, indicates that this enzyme plays a role in the development of intestine in newborn animals. Cathepsin D is also associated with human breast cancer metastasis (Rocheft, Acta Oncologica 31, 125-130 (1992)). Cathepsin E is located  
10 in the endoplasmic reticulum of some cells, such as erythrocyte and stomach mucosa cells. It has been applied in the processing of antigens in the immune cells.

Human aspartic proteases have important medical uses. The levels of the proenzymes of human pepsinogen and progastricsin present in the bloodstream and the ratio between the two levels is used in the diagnostic  
15 screening of human stomach cancer (Defize, *et al.*, Cancer 59, 952-958 (1987); Miki, *et al.*, Jpn. J. Cancer Res. 84, 1086-1090 (1993)) and ulcer (Miki, *et al.*, Adv. Exp. Med. Biol. 362, 139-143 (1995)). The secretion of procathepsin D is elevated in breast cancer tissue. Thus, the level of procathepsin D in breast cancer is used for clinical prognosis (Rocheft, Acta Oncologica 31, 125-130 (1992)). The analysis of renin in the  
20 diagnosis of hypertension is a routine clinical procedure (Brown *et al.*, Handbook of Hypertension 1, 278-323 Robertson, editor (Elsevier Science Publishers, Amsterdam, 1983)).

These examples establish that human aspartic proteases are related  
25 to human diseases and additional, previously unidentified aspartic proteases, are likely to have clinical applications.

It is therefore an object of the present invention to provide a previously unidentified aspartic protease.

It is a further object of the present invention to characterize and to  
30 clone the aspartic protease.

It is still another object of the present invention to identify the tissues in which the aspartic protease is expressed and applications in clinical chemistry and diagnostics.

### Summary of the Invention

5 A previously unknown aspartic protease capable of cleavage of proteins by hydrolysis, referred to herein as "napsin", has been cloned from a human liver library. Two cDNA clones have been cloned, sequenced and expressed. These encode isozymes of the protease, referred to as "napsin A" and "napsin B". One clone is unusual in that it  
10 does not include a stop codon but can be used to express protein. The gene has also been obtained and partially sequenced. A process for rapid purification of the enzyme using immobilized pepstatin has also been developed, and enzyme isolated from human kidney tissue. Polyclonal antibodies to the enzymes have been made which are also useful for  
15 isolation and detection of the enzyme.

Similarities to other aspartic proteases, especially cathepsin D, establish the usefulness of the enzyme in diagnostic assays as well as as a protease. Either or both the amount or type of napsin expressed in a particular tissue can be determined using labelled antibodies or nucleotide  
20 probes to the napsin.

### Brief Description of the Drawings

Figure 1 is the cDNA (SEQ ID No. 1) and putative amino acid sequence (SEQ ID No. 2) of human Napsin A. Characteristic active site elements (DTG) and Tyr75 are underlined. The RGD integrin binding  
25 motif is also underlined. Lysines at the carboxy terminus correspond to the poly-A region.

Figure 2a is a comparison of the human napsin A amino acid sequence with the amino acid sequences of mouse aspartic protease-like protein (Mori, et al., 1997) and human cathepsin D ("cath D"). Figure

2b is a schematic or dendrogram presentation of sequence relatedness between napsin and other human aspartic proteases.

Figure 3a is the genomic DNA (SEQ ID No. 3) of human Napsin A. Introns are indicated in lower-case letter, exons in upper case.

5 Putative amino acid sequence indicates position of intro-exon junctions.

Figure 3b is a schematic presentation of the human napsin A. The exons are shown as vertical bars with the numbering above. The double-headed arrows represent the areas where sequence was determined. The letters are positions of restriction sites where X is XhoI, B is BamHI, and  
10 E is EcoRI.

Figure 4 is the cDNA (SEQ ID No. 4) and putative amino acid sequence (SEQ ID No. 5) of human Napsin B. Characteristic active site elements (DTG) and Tyr75 are underlined. The RGD integrin binding motif is also underlined. Lysines at the carboxy terminus correspond to  
15 the poly-A region.

### Detailed Description of the Invention

#### I. Cloning and Expression of Napsin Isoforms.

##### A. Human Napsin A.

##### 1. *Cloning of cDNA encoding Napsin A.*

20 Clones identified by a homology search of the human cDNA sequence database of the Institute for Genome Research (Adams et al., Science 252, 1651-1656 (1991), reported to encode portions of cathepsin D, were obtained from the American Type Culture Collection, Rockville, MD. These are referred to as ATCC clone number 559204, 540096,  
25 346769, 351669, and 314203; Genbank numbers W19120, N45144, R18106, R11458, and T54068, respectively. Analysis of the sequences indicated these did not encode cathepsin D, and were not full length cDNAs. Primers were designed and used with PCR to obtain additional clones, using a human liver cDNA library as the template. The clones  
30 that were obtained include regions not present in the ATCC clones.

Since these clones together provided only about 600 bp of the cDNA, a longer cDNA clone was sought using 5' RACE PCR

(polymerase chain reaction), in which DNA from two separate human liver cDNA libraries cloned into  $\lambda$ gt10 was used as template and the primers were based on the near 5'-end sequence (AGGGCACA CTGAAGAAGTGGCATCTCC) (SEQ ID No. 5) and the sequence of the  $\lambda$ gt10 vector upstream from the insert in the forward direction (CTTTTGAGCAAGTTCAGCCTGGTTAAG) (SEQ ID No. 6). Two clones, pHL-1 (154 bp) and pHL-2 (288 bp) were obtained, one (pHL-2) of which extended the 5'-end sequence into the leader peptide region (Figure 1).

Human napsin A cDNA sequence lacks a stop codon from all clones obtained, yet all features otherwise indicate a functional aspartic protease, including intact active site elements, a conserved Tyr75 (pepsin numbering), and a pro-peptide of approximately 40 amino acids. Different from pepsin, the characteristic aspartic protease, napsin A contains a C-terminal extension, abundance of proline residues, and an RGD motif (integrin-binding motif) near the surface of the 3-D structure of napsin as judged by homologous crystal structures of mammalian aspartic proteases (i.e., pepsin and cathepsin D).

Several related cDNA clones of napsin were obtained by screening of a human liver cDNA library and the nucleotide sequences determined. These clones represent different parts of napsin messenger RNA. Spliced together, the nucleotide sequence encoding napsin A (SEQ ID No. 1) having the deduced amino acid sequence (SEQ ID No. 2) is shown in Figure 1.

## 2. *Expression of Recombinant Napsin A*

The cDNA of napsin A, including the leader peptide and the 3' untranslated region and a stretch of polyadenine, was PCR amplified with primers PLHNAP-FWD (SEQ ID No. 7)

(5'- AAGCTTATGTCTCCACCAACGCTGCTGCTACCCTTGCTGC)

and PLHNAP-REV (SEQ ID No. 8)

(5'- AAGCTTTTATTTTTTTTTTTTTTTTCAATGGAAATATTGG)

and cloned into the HindIII site of vector pLNCX for expression from the CMV promoter (Dusty Miller). Isolated plasmid was transformed into human kidney 293 cells (ATCC). Cells were recovered (8 - 120 mg) and lysed with 50 mM NaOAc, 20 mM zwittergent, pH 3.5 (NAZ buffer) with vortexing. Lysate was incubated on ice for 1 hour. The supernatant from centrifugation at 14,000 xg was employed directly for detection of expressed Napsin A by addition of a 40  $\mu$ l aliquot of pepstatin-A-agarose (Sigma). The sample was rotated in a 50 ml conical tube at 4°C for 1 week. The matrix was settled and washed twice with 20 ml of NAZ buffer, and three times with 20 mM Tris HCl, 0.5 M KCl, pH 8.2 (TK buffer). Final washes were performed with 20 mM Tris HCl, 50 mM NaCl, and 20 mM zwittergent, pH 9.5. The settled pepstatin-A-agarose (approximately 40  $\mu$ l) was mixed with 40  $\mu$ l of SDS- $\beta$ -mercaptoethanol sample buffer (NOVEX) and heated to 70°C for 10 minutes. Aliquots were applied to 10% Tricine SDS-PAGE (NOVEX) and transblotted to PVDF membranes using a Tris-Tricine buffer system. Membranes were either stained with amido black or blocked with 5% skim milk solution for immunochemical detection. Sections of membrane stained with amido black were excised and washed in sterile H<sub>2</sub>O for amino-terminal sequence analysis in an automated Protein Sequencer.

### 3. *Cloning of Genomic DNA.*

Genomic clones of human napsin were obtained by screening of a human genomic DNA library, cloned into bacterial artificial chromosomes (pBELO-BAC11) (Kim et al., Nucl. Acids Res. 20, 1083-1085 (1992)). The source of genomic DNA for the library was from 978SK and human sperm cell lines, and contained over 140,000 clones. Synthetic oligonucleotide probes were labelled with <sup>32</sup>P:

for primary screen Nap-3'

(GAGGGCGAGCGCGCGCCAGTCCCACTCGTGCGCCGCTCTTCATG  
TCCCCG) (SEQ ID No. 8),



and for secondary screening Nap-5'

(CCATCCCCTCAGTAGGTTTCAGGGTCCTGCGTCCAGGGTGGACTT  
GACGAA) (SEQ ID No. 9).

The screening was carried out at Research Genetics, Huntsville,  
Alabama. Two independent clones were isolated, both approximately 30  
kbp in length, and were cut with restriction enzyme and analyzed by  
pulse-field agarose gel electrophoresis. Fragments of interest were  
identified by Southern blotting, subcloned into pBlue, and sequenced.  
The genomic DNA of human Napsin A is shown in Figure 3A.

The human napsin A gene is encoded in 9 exons (Figure 3b). The  
exon/intron junctions are clearly defined by both the cDNA sequence and  
the junction motifs. The human napsin A coding region contains an open  
reading frame starting from the initiation codon ATG (nucleotide 1 in  
Figure 1) for about 1.2 kb to a polyA stretch in the cDNA sequences. As  
in the cDNA sequence of napsin A, the genomic exon sequence of napsin  
A do not contain an in-frame stop codon in the entire coding region  
before the polyA stretch. The absence of a stop codon in napsin A is  
confirmed. The absence of stop codon has not been observed for the gene  
of other mammalian proteins. The cDNA (thus the mRNA) of napsin A  
is present in different human tissues. It was of interest to see if napsin A  
gene is capable of expressing protein product. These results are described  
below.

#### B. Human Napsin B.

##### 1. *cDNA and gene structure.*

Clones 559204 and 163167 expressing human napsin B were  
obtained from ATCC and partially sequenced as described above. Figure  
4 displays the resulting full-length DNA sequence encoding Napsin B  
(SEQ ID No. 3) and the predicted amino acid sequence (SEQ ID No. 4).  
Nucleotides 1 - 1191 were obtained from genomic clones (described  
above for Napsin A) and from 1192 - 1910 from ATCC cDNA clones.  
The napsin B gene sequence is 92% identical to that of napsin A, and the  
putative protein sequence from each exhibits 91% identity. Similar to

napsin A, the deduced napsin B protein sequence possesses typical aspartic protease motifs, and the same c-terminal extension, RGD motif, and proline-rich regions as in the cDNA of napsin A (Fig. 4). Unlike the napsin A gene, napsin B gene has an in-frame stop codon.

5     **II.     Isolation and Characterization of Napsin Protein.**

          The comparison of the napsin A sequence with three other human aspartic protease proenzymes is shown in Figure 2A. It is clear that napsin is related to human cathepsin D, and is similar to mouse aspartic protease-like protein, but the differences are readily apparent. The  
10    relationship to other human aspartic proteases is further analyzed in Figure 2B, which is a diagram of degree of relatedness and also presents the percentage of identical residues. Clearly, by both criteria, napsin differs as much from other aspartic proteases as they differ from one another.

15           In addition to the sequence similarity to the other human aspartic proteases, the conclusion that napsin is an aspartic protease is drawn from the following observations. (a) The critical active site aspartic residues at positions 32 and 215 are present in the conserved DTG sequences. (b)  
          The presence of Tyr-75 (Y) and some conserved residues around it  
20    indicate a functional 'flap' which is characteristic of aspartic proteases. (c) The pro region corresponding to residues 1p to 44p is present in napsin, indicating that it is a proenzyme of the aspartic protease and is capable of activation.

          An RGD sequence is found at position 315 to 317 (porcine pepsin  
25    residue numbers by convention). This motif has been shown to be important in integrin bonding which is related to the regulation of cellular functions such as cell cycle, hemostasis, inflammation and cell proliferation. This sequence may have particular functional meaning for napsin.

30           **2.     Immunochemical Detection of Napsin A.**

          A napsin-specific polyclonal antiserum was produced using the following procedure. An 18 amino acid epitope of Napsin A which was

synthesized as a multiple antigenic peptide (MAP) on a poly-lysine backbone by the Molecular Biology Resource Facility (OUHSC). This epitope (MKSGARVGLARARPRG) was common to both napsin A and B, and sufficiently dissimilar from cathepsin D, their closest homolog.

5 This region is likely to be located on the surface of Napsin A as determined from the cathepsin D crystal structure coordinates (Erickson, 1993). Aliquots of 1 mg in 1 ml of H<sub>2</sub>O were used to immunize goats (Hybridoma Lab, Oklahoma Medical Research Foundation). Serum collected was ammonium sulfate precipitated multiple times (Antibodies  
10 Lab manual) and affinity purified using the Napsin A MAP coupled to affi-gel 10 (BioRad). This anti sera was used at 1:5000 dilution in the detection of Napsin A on PVDF membranes transblotted from SDS-PAGE gels (NOVEX). The ECL system (Pierce) was used for detection of primary antibody.

15 Immunoblots of recombinant Napsin A sample from human kidney 293 cells prepared as described above detected Napsin A. These results show expression of napsin A gene produced an immunospecific band which migrated in SDS-polyacrylamide electrophoresis with a similar mobility to that of napsin B. Thus, despite of the absence of a stop codon  
20 in napsin A, its protein is correctly expressed in a human cell line. The fact that this napsin A protein was recovered from the pepstatin-affinity column suggests that the presence of an active site similar to all aspartic proteases.

### 3. *Detection of Napsin B in Human Tissue and Cell*

#### 25 *Lines*

Sections of approximately 8 grams of human kidney cortex (Cooperative Human Tissue Network, National Cancer Institute, NIH) were homogenized in a Waring blender in buffer composed of 20 mM Tris HCl, 50 mM NaCl, 20 mM zwittergent, and 1  $\mu$ M each of TPCK,  
30 TLCK, and EDTA, pH 7.5 (buffer TZ). The homogenate was made 40% ammonium sulfate with gentle stirring, and centrifuged 10,000 xg. The resulting supernatant was made 70% ammonium sulfate and centrifuged

10,000 xg. The material insoluble in 70% ammonium sulfate (the 40-70% cut) was dissolved in 15 ml of buffer TZ and made pH 4.0 with 30 ml of NAZ buffer. Following incubation on ice for 1 hour, the sample was centrifuged at 14,000 x g. To the resulting supernatant, a 0.1 ml  
5 aliquot of pepstatin-A-agarose (Sigma) was added. Detection of napsin B in cell lines followed the procedure outlined above for detection of recombinant napsin A.

Napsin B was detected in tissue samples of human kidney cortex and in the human kidney cell line Hut-78: human kidney (0-40%  
10 ammonium sulfate cut); human kidney (40-70% cut); Hut-78 cells, in apparently four forms. In the 0-40% ammonium sulfate cut, a single-chain protease of 50-54 kDa with a heterogeneous amino terminus sequence derived from the protein sequence of SPGDKPIFVPLSNYR (with other termini at Asp4 and Lys5) was detected. These N-terminal  
15 sequences agreed well with the predicted activation cleavage site in pronapsin B by comparing to the activation cleavage sites in homologous procathepsin D and other aspartic protease zymogens. In the 40-70% ammonium sulfate cut, three forms were detected. A 46-50 kDa single chain form, and two two-chain forms. The 46-50 kDa band produced the  
20 same heterogeneous sequence Napsin B sequence as obtained for the larger molecular weight band in the 40% ammonium sulfate cut. The two lower molecular weight fragments of approximately 8 and 4 kDa produced the same amino-terminal sequence  
(VRLCLSGFQALDVPPPAGPF) corresponding to the C-terminal region of  
25 Napsin B. A prominent 40 kDa band of the transblotted preparation was sequenced, and produced the same heterogeneous amino terminal sequence as the 46-50 kDa band, indicating two species of two-chain Napsin B: an 8 kDa and 40 kDa as well as a 4 kDa and a 40 kDa species.

### 30 III. Applications of Napsin.

A variety of clinical and diagnostic uses for the enzyme can be designed based on analogy to the uses of the related aspartic proteases.

The proteins, nucleotide molecules, and methods for isolation and use thereof have a wide variety of applications, particularly in diagnostic applications. Since aspartic proteases are well known to be correlated with certain disorders, such as breast cancer and high blood pressure, and napsin is expressed in the kidney, measurement of the levels and/or types of napsin expressed in tissue, especially kidney, can be correlated with the presence and severity of disorders. The recombinant DNA and reagents derived therefrom can be used to assay for napsin expression in healthy and in people inflicted with illness. Napsin sequences can be used to track the presence of napsin genes in patients for possible linkage to diseases.

A. Diagnostic Applications

The amount of napsin can be determined using standard screening techniques, ranging from isolation of napsin from the tissue, using for example immobilized anti-napsin (or anti-napsin A or anti-napsin B) or pepstatin, to detection and quantification with labelled antibodies, to determination of the amount of mRNA transcribed in the tissue, using labelled nucleotide probes.

*Antibody Production*

Polyclonal antibodies were produced using standard techniques for immunization of an animal with purified protein in combination with an adjuvant such as Freund's adjuvant. Monoclonal antibodies can also be prepared using standard techniques, for example, by immunizing mice until the antibody titer is sufficiently high, isolating the spleen and doing a fusion, and then screening the hybridomas for those producing the antibodies of interest. These can be antibodies reactive with any napsin, or reactive with napsin A but not B and vice versa.

Humanized antibodies for therapeutic applications, and recombinant antibody fragments can also be generated using standard methodology. A humanized antibody is one in which only the antigen-recognition sites or complementarity-determining hypervariable regions (CDRs) are of non-human origin, and all framework regions (FR) of

variable domains are products of human genes. In one method of humanization of an animal monoclonal anti-idiotypic antibody, RPAS is combined with the CDR grafting method described by Daugherty *et al.*, Nucl. Acids Res., 19:2471-2476 (1991). Briefly, the variable region

5 DNA of a selected animal recombinant anti-idiotypic ScFv is sequenced by the method of Clackson, T., *et al.*, Nature, 352:624-688 (1991). Using this sequence, animal CDRs are distinguished from animal framework regions (FR) based on locations of the CDRs in known sequences of animal variable genes. Kabat, H.A., *et al.*, Sequences of

10 Proteins of Immunological Interest, 4th Ed. (U.S. Dept. Health and Human Services, Bethesda, MD, 1987). Once the animal CDRs and FR are identified, the CDRs are grafted onto human heavy chain variable region framework by the use of synthetic oligonucleotides and polymerase chain reaction (PCR) recombination. Codons for the animal heavy chain

15 CDRs, as well as the available human heavy chain variable region framework, are built in four (each 100 bases long) oligonucleotides. Using PCR, a grafted DNA sequence of 400 bases is formed that encodes for the recombinant animal CDR/human heavy chain FR protection. The expression of recombinant CDR-grafted immunoglobulin gene is

20 accomplished by its transfection into human 293 cells (transformed primary embryonic kidney cells, commercially available from American Type Culture Collection, Rockville, MD 20852) which secrete fully grafted antibody. See, *e.g.*, Daugherty, B.L., *et al.*, Nucl. Acids Res., 19:2471-2476, 1991. Alternatively, humanized ScFv is expressed on the

25 surface of bacteriophage and produced in *E. coli* as in the RPAS method described below.

Pharmacia's (Pharmacia LKB Biotechnology, Sweden)

"Recombinant Phage Antibody System" (RPAS) may be used for this purpose. In the RPAS, antibody variable heavy and light chain genes are

30 separately amplified from the hybridoma mRNA and cloned into an expression vector. The heavy and light chain domains are co-expressed on the same polypeptide chain after joining with a short linker DNA

which codes for a flexible peptide. This assembly generates a single-chain Fv fragment (ScFv) which incorporates the complete antigen-binding domain of the antibody. Using the antigen-driven screening system, the ScFv with binding characteristics equivalent to those of the original monoclonal antibody is selected [See, e.g., McCafferty, J., *et al.*, Nature, 348:552-554 (1990); Clackson, T., *et al.*, Nature, 352:624-688 (1991). The recombinant ScFv includes a considerably smaller number of epitopes than the intact monoclonal antibody, and thereby represents a much weaker immunogenic stimulus when injected into humans. An intravenous injection of ScFv into humans is, therefore, expected to be more efficient and immunologically tolerable in comparison with currently used whole monoclonal antibodies [Norman, D.J., *et al.*, Transplant Proc., 25, suppl. 1:89-93 (1993).

#### *Nucleotide Probes*

Nucleotide probes can be used to screen for napsin expression or the types and/or ratios of isoforms present. These can be cDNA sequences or other molecules designed based on the sequences reported herein, or which are obtained using standard techniques from libraries generated from different cell types or species. It is understood that while the sequence reported here is of human origin, the same proteases will be present in other species of animals, and will vary to some degree in both the amino acid sequence and the nucleotide sequence. Napsin is referred to herein as an aspartic protease having the naturally occurring amino acid sequence from human or other animals, or a composite sequence constructed by substitution of amino acids from one species into another, at the equivalent position, other than at the active site, discussed above. A nucleotide molecule encoding napsin can be naturally occurring, as described herein, or designed and made synthetically based on the amino acid sequence. Moreover, since at least two isoforms have been identified, it is expected that additional isoforms will be found in tissues other than kidney or liver. These isoforms are intended to encompass within the term "napsin".

Nucleotide molecules can be used to assay for amount, type or a combination thereof, using standard diagnostic techniques. In general, probes will include a segment from a DNA encoding napsin of at least fourteen nucleotides, which should be sufficient to provide specificity under standard hybridization conditions, and even more so under stringent conditions. Reaction conditions for hybridization of an oligonucleotide probe or primer to a nucleic acid sequence vary from oligonucleotide to oligonucleotide, depending on factors such as oligonucleotide length, the number of G and C nucleotides, and the composition of the buffer utilized in the hybridization reaction. Moderately stringent hybridization conditions are generally understood by those skilled in the art as conditions approximately 25°C below the melting temperature of a perfectly base-paired double-stranded DNA. Higher specificity is generally achieved by employing incubation conditions having higher temperatures, in other words more stringent conditions. In general, the longer the sequence or higher the G and C content, the higher the temperature and/or salt concentration required. Chapter 11 of the laboratory manual of Sambrook et al., MOLECULAR CLONING: A LABORATORY MANUAL, second edition, Cold Spring Harbor Laboratory Press, New York (1990), describes hybridization conditions for oligonucleotide probes and primers in great detail, including a description of the factors involved and the level of stringency necessary to guarantee hybridization with specificity. Below 10 nucleotides, hybridized systems are not stable and will begin to denature above 20°C. Above 100,000 nucleotides, one finds that hybridization (renaturation) becomes a much slower and incomplete process, as described in greater detail in the text MOLECULAR GENETICS, Stent, G.S. and R. Calender, pp. 213-219 (1971). Ideally, the probe should be from 20 to 10,000 nucleotides. Smaller nucleotide sequences (20-100) lend themselves to production by automated organic synthetic techniques. Sequences from 100-10,000 nucleotides can be obtained from appropriate restriction endonuclease treatments. The labeling of the smaller probes with the relatively bulky



chemiluminescent moieties may in some cases interfere with the hybridization process.

### *Labels*

Both antibodies and nucleotide molecules can be labelled with  
5 standard techniques, for example, with radiolabels, fluorescent labels, chemiluminescent labels, dyes, enzymes, and other means for detection, such as magnetic particles. For example, selective labeling of the active site with fluorescein can be performed by the method of Bock (Bock, P.E. (1988) Biochemistry 27, 6633-6639). In brief, a blocking agent is  
10 reacted with enzyme for 1 hour at room temperature. After dialysis, the covalently modified enzyme is incubated at room temperature for one hour with 200  $\mu$ M 5-(iodoacetamido)fluorescein (Molecular Probes). Free fluorescein is removed by gel filtration on a PD-10 column (Pharmacia). With this method, each molecule of fluoresceinated enzyme contains a  
15 single dye at the active site and hence all of the fluorescent molecules behave identically. Alternatively, iodogen (Pierce) can be used to radiolabel enzyme with Na[ $^{125}$ I] (Amersham) according to the manufacture's protocol. Free  $^{125}$ I can be removed by gel filtration on a PD-10 column.

### *Recombinant Protein*

Recombinant proteins, and fragments thereof, are useful as controls in diagnostic methods. The cDNA and gene sequences of napsin A were determined. The DNA was expressed in a recombinant system (human cell line) and the activity of the enzyme characterized. The  
25 cDNA and gene sequences of napsin B were determined. The proteins can be used as standards, or as discussed below, therapeutically as aspartic proteases and in studies of enzyme behavior. The expression of recombinant proteins from a cDNA without stop codon may offer certain advantages.

### *Procedures for isolation of Napsin*

30 Antibodies and nucleotide probes are primarily useful in the detection of napsin, or its isoforms. In some cases it may also be useful

to isolate the purified protein. As described above, a procedure was devised to bind napsin A and napsin B on to a pepstatin-affinity column. Immobilized pepstatin can be used to purify either naturally occurring, or recombinant, napsin, from tissues in which it is expressed, for diagnostic applications.

B. Enzyme Applications.

The aspartic proteases may be useful in applications similar to those for which cathepsin D are used. Clinically, it may be advantageous to transfect, even transiently, the gene encoding napsin to treat disorders in which the individual is deficient in the protease, or to transfect an antisense, targeted ribozyme or ribozyme guide sequence, or triple helix to prevent or decrease enzyme expression, in individuals with disorders characterized by elevated levels of enzyme.

We claim:

1. An isolated napsin.
2. The napsin of claim 1 wherein the protein is isoform A.
3. The napsin of claim 2 having the amino acid sequence of SEQ ID No. 2.
4. The napsin of claim 2 encoded by SEQ ID No. 1.
5. The napsin of claim 1 wherein the protein is isoform B.
6. The napsin of claim 5 having the amino acid sequence of SEQ ID No. 4.
7. The napsin of claim 5 encoded by SEQ ID No. 3.
8. An isolated nucleotide molecule encoding napsin.
9. The molecule of claim 8 encoding napsin A.
10. The molecule of claim 10 as depicted by SEQ ID No. 1.
11. The molecule of claim 8 encoding napsin B.
12. The molecule of claim 11 as depicted by SEQ ID No. 3.
13. The molecule of claim 8 or a portion of at least fourteen nucleotides unique to napsin labelled with a detectable label.
14. A method for isolating napsin comprising isolating the protein bound to immobilized pepstatin in an tissue extract.
15. The method of claim 14 wherein the tissue is kidney cells.
16. A method for detecting the amount or type of napsin present in a tissue comprising reacting the tissue with a labelled nucleotide molecule probe specifically hybridizing to DNA or RNA encoding napsin, or reacting the tissue with a labelled antibody specifically immunoreactive with napsin.
17. The method of claim 16 wherein the tissue is screened for the level of expression of both napsin A and napsin B.
18. The method of claim 16 wherein the amount or type of napsin present in the tissue is compared to the amount or type of napsin present in a normal control tissue.
19. An antibody specifically immunoreactive with napsin.

20. The antibody of claim 19 wherein the antibody is immunoreactive with either napsin A or napsin B.

1 ATGTCCTCCACCACCGCTgctgCTcccCTtGCTGCTGctGCTGcCTcTGCTGAATGTGGAGCCTGCTGGGGCCACACTGATCCGGATCCcTcTTcGTCAAG 100  
M S P P P L L L L P L L L L L L P L L N V E P A G A J L I R I P L R Q V

101 TCCACCCCTGGACGCGAGGACCCTGAACCTACTGAGGGGATGGGAAAAACCAGCAGAGCTCCCAAGTTGGGGGCCCATCCCTTGGGGACAAGCCTGCCTC 200  
H P G R R T L N L L R G W G K P A E L P K L G A P S P G D K P A S

201 GGTACCTCTCTCCAAATTCCTGGATGCCCAgTATTTTGGGgaAATTGggctgGGAACGCCTCCACAAAACTTCACTGTTGCCTTTGACACTGGCTCCTCC 300  
V P L S K F L D A Q Y F G E I G L G T P P Q N F T V A F D T G S S

301 AATCTCTGGGTCCCGTCCAGGAGATGCCACTTCTTCAGTGTGCCCTGCTGTTCCACCACCGCTTCAATCCCAATGCCTcCAGCTCCTTCAAGCCCACTG 400  
N L W V P S R R C H F F S V P C W F H H R F N P N A S S S S F K P S G

401 GGACEAAGTTTGCCATTCACTATGGAACCTGGCGGGTAGATGGAATCCTGAGTgAGGACAAGCTGACTATtGGTGGAAATCaAGCGTGCATCCGTGATTTT 500  
T K F A I Q Y G T G R V D G I L S E D K L T I G G I K G A S V I F

501 CggGgAagcTCTGTGGGAATCCAGcctGGTCTTCACTGTTTCCCGCCCGATGGGATATTGGGCTCGGTTTTCCCATTTCTGTCTGTGGAAGGAgTTCGG 600  
G E A L W E S S L V F T V S R P D G I L G L G F P I L S V E G V R

601 CCCCCGCTGGATGTACTGGTGGAGCAGGGGCTATTGGaTAAGCCTGTCTTCTCCTTTTACTTcACAGGgacCCTGAAGTGGCTGATGGAgGAgAgCTGG 700  
P P L D V L V E Q G L L D K P V F S F Y F W R D P E V A D G G G E L V

701 TcTggggGGGcTCagACCCGGCACACTACATCCACCCCTCACCTTcGTGCCAGTCACAGTCCCCGCTAcTGGCAGATCCACATGGAgCGTGTgAAgGT 800  
L G G S D P A H Y I P P L T F V P V T V P A Y W Q I H M E R V K V

801 GGGETCACGGCTgActetcTGTGCCAgGGCTGTGCTGCCATcCTGGATACAgGCACACCTGTcATcGTAgGACCCActgAgGAgATCCGGGCCCTGCAT 900  
G S R L T L C A Q G C A A I L D T G T P V I V G P T E E I R A L H

901 GCAGCCATTGGGGGAATCCCTTGTGCTGGGTGGGAgTAcATCATCCGGTGCTCagAAATCCCAAAgCtCCCCGCAgTcTCAcTCCTCATTGGGGGGTcT 1000  
A A I G G I P L L A G E Y I I R C S E I P K L P A V S L L I G G V W

1001 GGTTTAATCTCACGgCCCAgGAATAcGTcATCCAGTTTgCtCAGGGTGAcGTCCGcCtCtGCTTGTcCGGCTCCGGgCCTTGGACATCGCTTCGCCTCC 1100  
F N L T A Q D Y V I Q F A Q G D V R L C L S G F R A L D I A S P P

1101 AGTACCTGTGTGGATCCTCGGCGACGTTTTCTTgGGGGGTATGTGACCGTCTTCGACCGGGGACATGAAGAGCGGCGCcCgAgTGGGAcTGGCGCGC 1200  
V P V W I L G D V F L G A Y V T V F D R G D M K S G A R V G L A R

1201 GCTCGCCCTCgCGGAgCGGACCTGGGAAGGCGCGAGACCGCGCAGGCGCAGTACCGGGGTGCCGCCAGGTGATGCGCATGCGCACCGGGTAGCCGAGC 1300  
A R P R G A D L G R R E T A Q A Q Y R G C R P G D A H A H R V A E L

1301 TcgcgCTACTCAGTAAAAATCCAATATTTCATTGAAAAAAAAAAAAAAAAAAAA 1353  
A L L S K N P I F P L K K K K K K

FIGURE 1

	-60	-50	-40	-30	-20
H-Napsin	MSPPPILLPL	LLLLPLINVE	PAGATLIRIP	LROVHPGRRT	INLLRGWGK.
M-KAP	MSP...LLL	LLCLLLGNLE	PEERKLIRVP	LQRIHLGHRI	INPLNGWEQ.
H-CathD	.MQPSSLLPL	ALCL....LA	APASALVRIP	LHKFTSIRRT	MSEVGGSVED
	-10	1	10	20	30
H-Napsin	...PAELPKL	GAPSPGDKPA	SVP...LSKFL	DAQYFGEIGL	GTPPONFTVA
M-KAP	...LAELSR.	.TSTSCGNPS	FVP...LSKFM	NTQYFGTIGL	GTPPONFTVV
H-CathD	LIAGPVS KY	SQAVPAVTEG	PIPEVLKNYM	DAQYGEIGI	GTPPCFTVV
	40	50	60	70	
H-Napsin	FDTGSSNLWV	PSRRCHFFSV	PCWFHHRFNP	NASSSFKPSG	TKFAIQYGTG
M-KAP	FDTGSSNLWV	PSTRCHFFSL	ACWFHHRFNP	KASSSFRPNG	TKFAIQYGTG
H-CathD	FDTGSSNLWV	PSIHCKLLDI	ACWIHHKYN	DKSSTVVKNG	TSFDIHVSG
	80	90	100	110	
H-Napsin	RVDGILSEDK	LTI.....	....GGIKGA	SVIFGEALWE	SSLVFTVSRP
M-KAP	RLSGILSQDN	LTI.....	....GGIHDA	FVTFGEALWE	PSLTFALAHF
H-CathD	SLSGYLSQDT	VSVPCQSASS	ASALGGVKVE	RQVFGAATKQ	PGITFIAAKF
	120	130	140	150	160
H-Napsin	DGILGLGFPI	LSVEGVRPPL	DVLVEQGILLD	KPVFSFYFNR	DPÉVADGGEL
M-KAP	DGILGLGFPT	LAVGGVQPPL	DAMVEQGILLE	KPVFSFYLN	DSEGSDDGEL
H-CathD	DGILGMAYPR	ISVNNVLPVF	DNLMOQKLVD	QNIFSFYLSR	DPDAQPGGEL
	170	180	190	200	210
H-Napsin	VLGGSDPAHY	IPPLTFVPVT	VPAYWQIHME	RVKVGSRLTL	CAQGCAATLD
M-KAP	VLGGSDPAHY	VPPLTFIPVT	IPAYWQVHME	SVKVGTGLSL	CAQGCATLD
H-CathD	MLGGTDSKY	KGSLSYLNV	RKAYWQVHLD	QVEVASGLTL	CKEGCEATVD
	220	230	240	250	260
H-Napsin	TGTPVIVGPT	EEIRALHAAI	GGIPLLAGEV	IIRCSEIPKL	PAVSLLIGGV
M-KAP	TGTSLLTGPS	EEIRALNKAI	GGYPFLNGQY	FIQCSKTPTL	PPVSFHLGGV
H-CathD	TGTSMLVGPV	DEVRELQKAI	GAVPLIQGEV	MIPCEKVSTL	PATLKLGGK
	270	280	290	300	310
H-Napsin	WFNLTAQDYV	IQFAQGDVRL	CLSGFRALDI	ASPPVPVWIL	GDVFLGAYVT
M-KAP	WFNLTGQDYV	IQDLQSDVGL	CLLGFOALDI	PKPAGPLWIL	GDVFLGPYVA
H-CathD	GYKLSPEDYT	LKVSQAGKTL	CLSGFMGMDI	PPPSGPLWIL	GDVFIGRYYT
	320	326	330	340	350
H-Napsin	VFDRGDMKSG	ARVGLARARP	RGADLGRRET	AQAQYRGCRP	GDAHAHRVAE
M-KAP	VFDRGDKNVG	PRVGLARAQS	RSTDRAERT	TQAQFFKRRP	G.....
H-CathD	VFDRDN....	NRVGFAEAAR	L.....	.....	.....
	360	370			
H-Napsin	LALLSKNPIF	PLKKKKKK....			
M-KAP	.....	.....			
H-CathD	.....	.....			

FIGURE 2A

3/5

ATGCTCCACCACCGTCC  
M S P P P L L

10 20 30  
20 TCGTACCCCTTCTGCTGCTGCTGCTGCTGAATGTGGAGCCTGCTGGGCCCCACTGATCCGgtatggtgacccca.....ttttcataccctacagGATCCCTTTC  
L P L L L L L P L L N V E P A G A T L I R I P L R

40 50 60 70  
95 GTCAAGTCCACCCTGGACGAGGACCTGAACCTACTGAGGGGATGGGAAAAACAGCAGAGCTCCCAAGTTCGGGCCCCATCCCTGGGGACAAGCCTGCCTGGGTACCTCTCTCA  
Q V N P G R R T L N L L R G W G K P A E L P K L G A P S P G D K P A S V P L S K

80 90  
215 AATTCCTGGAgtgagtcacagccctacaca.....ctcttttttgcctctctcagGCCAGTATTTTGGGAAATGGGTGGGAACGCTCCACAAACTTCACTGTTGCTTTGACA  
F L D A O Y F G E I G L G T P P Q N F T V A F D I

100 110 120  
290 CTGGCTCTCCAATCTCTGGGTCCCGTCCAGGAGATGCCACTTCTTCAGTGTCCCTGCTgtgagcttctatgtgggaga....ccctctgacttctgacctagGGTCCACCACCGCT  
G S S M L W V P S R R C H F F S V P C F M H R F

130 140 150  
365 TCAATCCCAATGCTCCAGCTCCTTCAAGCCAGTGGGACCAAGTTGCCATTCACTATGGAATCGGCGGTAGATGGAATCCTGAGTGAGGACAAGCTGACTgtgagtgagcctttgac  
H P N A S S S F K P S G T K F A I O Y G T G R V D G I L S E D K L T

160 170 180  
469 tcaag.....acatctcaatctacccctagATTGGTGAATCAAGGGTGCATCCCTGATTTTGGGAAAGCTCTGTGGGAATCCAGCCTGGTCTTCACTGTTTCCCGCCCCGATGGCATAI  
I G G I K G A S V I F G E A L V E S S L V F T V S R P D G I L

190 200 210 220  
560 TGGGCTCGGTTTTCCCATTTCTGTGTGGAAGGAGTTCGGCCCCGCTGGATGACTGCTGGAGCAGGGCTATGGATAAGCCTGCTTCTCTCTTTACTTCAACAGgtactgggaaag  
G L G F P I L S V E G V R P P L D V L V E O G L L D K P V F S F Y F H R

230 240 250  
669 gtgcacct.....gtacactntgcccctgcagGGGACCCTGAAGTGGCTGATGGAGGAGAGCTGGTCTCGGGGCTCAGACCCGGACACTACATCCACCCCTCACCTTCTGTGCCAG  
D P E V A D G G E L V L G G S D P A N Y I P P L T F V P V

260 270  
755 TCACAGTCCCCGCTACTGGCAGATCCACATGGAGCGgtgaggacttgggtctctg.....actgcttcttccccctcagTGTGAAGGTGGGTCACGGTCACTCTCTGTGCCACG  
T V P A Y U O I H M E R V K V G S R L T L C A O C

280 290 300 310  
830 GGTGTGCTGCCATCCTGGATACAGGCACACTGTATGTAAGGACCACTGAGGAGATCCGGCCCTGCATGCCCATTCGGGGAATCCCTTGTGGCTGGGAGgtgagttccccag  
C A A I L D T G T P V I V G P T E E I R A L N A A I G G I P L L A G E

320 330 340  
937 tctct....ttgttctctctctccaccagTACATCATCCGTGCTCAGAAATCCAAAGCTCCCGCAGTCTCACTCTCAATTCGGGGGTCTGGTTTAAATCTACGGCCCAAGATTAAG  
Y I I R C S E I P K L P A V S L L I G G V U F N L I A O D T V

350 360  
1028 TCAICAGgtagggtgctgctcatabga....gcccgccttgtgcctttgagTTTGTCAAGGTGACGTCGCTCTGCTTGTCCGGCTTCGGGCTTGGACATTTTGTCTTGTG  
I O F A O G D V R L C L S G I R A L D T A S D P R V

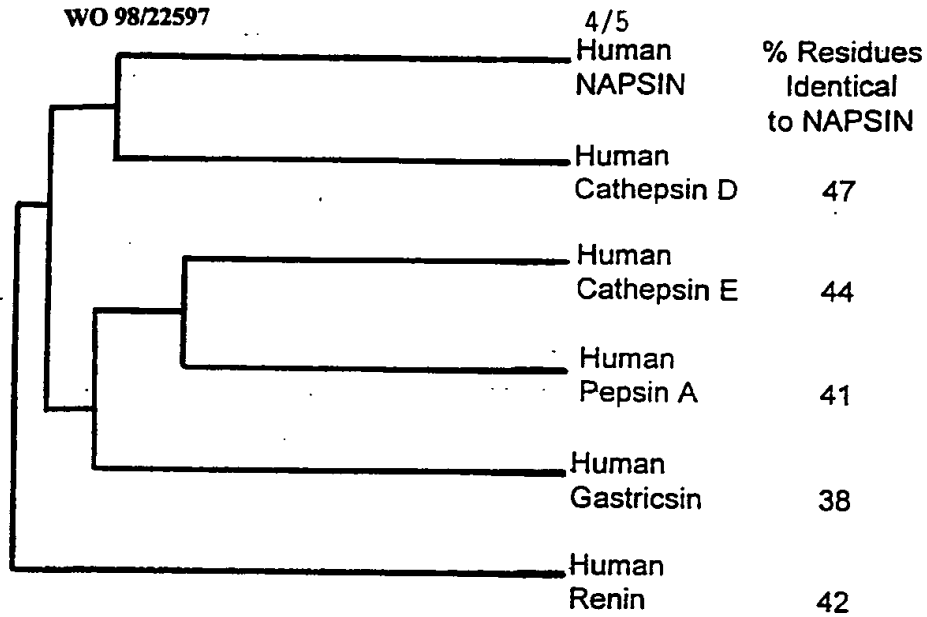
370 380 390 400  
1103 TACCTGTGTGATCCTCGGACGTTTTCTTGGGGGATGTGACCGTCTTCGACCCGGGACATCAAGACCCGGGACAGTGGGACTGGGCGGCTCGGCTTCGGGAGGGGAC  
P V W I L G D V F L G A T V T V F D R G D M K S G A R V G L A R A R P R G A D L

410 420 430 440  
1223 TGGGAAGGCGGAGACCCGCGAGTACCGGGTGGCGCCAGGTGATGCGCATGGCAGCGGTAGCGGCTAGCGGCTACTCAGTAAAAATCCAATTTCCATIGAACGAC  
G R R E T A O A O Y R G C R P G D A N A M R V A E L A L L S K N P I F P L

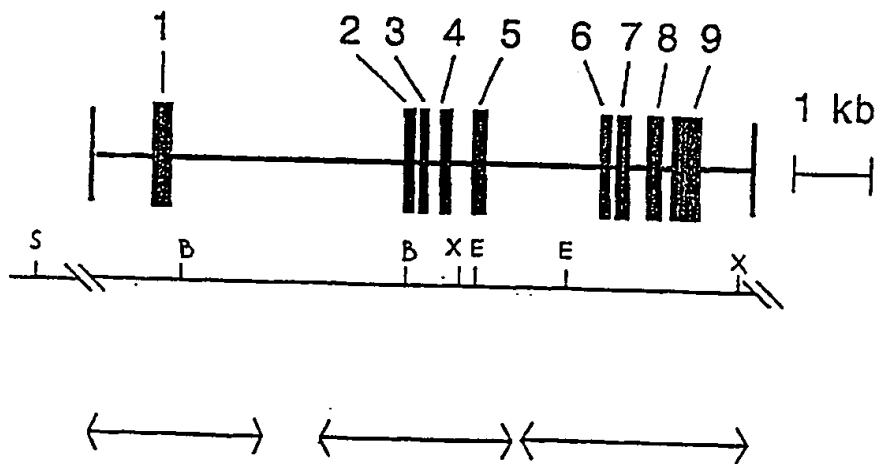
FIGURE 3A

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**FIGURE 2B**  
A dendrogram presentation of sequence relatedness between hapsin and other human aspartic proteinases.



**FIGURE 3B**



FIGURE 4

<p>(51) International Patent Classification 6 : C12N 15/57, 9/64, C12P 21/00, C07K 16/40, C12N 15/11, C12Q 1/68, G01N 33/50</p>	<p>A3</p>	<p>(11) International Publication Number: <b>WO 98/22597</b></p> <p>(43) International Publication Date: 28 May 1998 (28.05.98)</p>
<p>(21) International Application Number: PCT/US97/21684</p> <p>(22) International Filing Date: 20 November 1997 (20.11.97)</p> <p>(30) Priority Data: 60/031,196 20 November 1996 (20.11.96) US 60/046,126 9 May 1997 (09.05.97) US</p> <p>(71) Applicant: OKLAHOMA MEDICAL RESEARCH FOUNDATION [US/US]; 825 N.E. Thirteenth Street, Oklahoma City, OK 73104 (US).</p> <p>(72) Inventors: KEOLSCH, Gerald; 11617 Roxboro Avenue, Oklahoma City, OK 73162 (US). LIN, Xinli; 1201 Charlton, Edmond, OK 73003 (US). TANG, Jordan, J., N.; 1204 Leawood Drive, Edmond, OK 73034 (US).</p> <p>(74) Agents: PABST, Patrea, L. et al.; Arnall Golden &amp; Gregory, 2800 One Atlantic Center, 1201 West Peachtree Street, Atlanta, GA 30309-3450 (US).</p>	<p>(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).</p> <p><b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 9 July 1998 (09.07.98)</p>	
<p>(54) Title: CLONING AND CHARACTERIZATION OF NAPSIN, AN ASPARTIC PROTEASE</p>		
<p>(57) Abstract</p> <p>A previously unknown aspartic protease capable of cleavage of proteins by hydrolysis, referred to herein as "napsin", has been cloned from a human liver library. Two cDNA clones have been cloned, sequenced and expressed. These encode isozymes of the protease, referred to as "napsin A" and "napsin B". The gene has also been obtained and partially sequenced. A process for rapid purification of the enzyme using immobilized pepstatin has also been developed, and enzyme isolated from human kidney tissue. Polyclonal antibodies to the enzymes have been made which are also useful for isolation and detection of the enzyme. Similarities to other aspartic proteases, especially cathepsin D, establish the usefulness of the enzyme in diagnostic assays as well as a protease. Either or both the amount or type of napsin expressed in a particular tissue can be determined using labelled antibodies or nucleotide probes to the napsin.</p>		

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## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/57 C12N9/64 C12P21/00 C07K16/40 C12N15/11  
C12Q1/68 G01N33/50

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## B. FIELDS SEARCHED

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IPC 6 C12N C12P C07K C12Q G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	MORI K ET AL: "MOLECULAR CLONING OF A NOVEL MOUSE ASPARTIC PROTEASE-LIKE PROTEIN THAT IS EXPRESSED ABUNDANTLY IN THE KIDNEY" FEBS LETTERS, vol. 401, no. 2/03, 20 January 1997, pages 218-222, XP002052496 cited in the application see figures 1,3,4	1-20
E	WO 98 13484 A (INCYTE PHARMA INC ;COLEMAN ROGER (US); BANDMAN OLGA (US)) 2 April 1998 see sequence ID No 3	1,5-7, 11,12
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☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 May 1998

Date of mailing of the international search report

25/05/1998

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E	WO 98 11236 A (HILL JEFFREY ;KAY JOHN (GB); UNIV WALES (GB); SMITHKLINE BEECHAM P) 19 March 1998 see sequence ID No 1 ---	1-4,8-10
X	EMBL Database entry HS06826 Accession number T54068; 28 Feb 1995 HILLIER L et AL: Homo sapiens cDNA clone 74370 5' similar to gb:J05036, cathepsin E precursor (human) XP002064618 see sequenc3 ---	13
A	FAUST PH L ET AL: "CLONING AND SEQUENCE ANALYSIS OF CDNA FOR HUMAN CATHEPSIN D" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 82, August 1985, pages 4910-4914, XP002052494 -----	

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9813484 A	02-04-1998	NONE	
WO 9811236 A	19-03-1998	NONE	

Reviewed by: KGW on 4/3/01Patent#/author: WO 98/22597Keywords Aspartic proteaseNapsin

Review (if necessary)

Identifies new aspartic protease, "napsin"  
present in liver

Perceived significance:

Irrelevant / Somewhat significant / Moderately significant / Highly significant

1 2 3 4 5 6 7 8 9 10Reviewed by: dmh on 4/3/01Patent#/author: WO 98/22597Keywords DNA sequenceNapsin

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Perceived significance:

Irrelevant / Somewhat significant / Moderately significant / Highly significant

1 2 3 4 5 6 7 8 9 10